702 cm^{-1}; MS m/z 265 ($M^+ - C_2H_5$, 54), 205 (36), 149 (100), 145 (81), 115 (32), 91 (27), 77 (34), 59 (40). Anal. Calcd for $C_{16}H_{22}O_5$: C, 65.29; H, 7.53. Found: C, 65.03; H, 7.64. **5-Ethyl-3-(methoxycarbonyl)-5-phenyl-γ-butyrolactone [10 (R′ = OMe, R″ = Et, Ar = C_6H_5)]** was also recovered in 59% of yield (1.3:1 mixture of two diastereoisomers): ^{1}H NMR (200 MHz) referred to the main stereoisomer δ 7.3 (tight m, 5 H), 3.78 (s, 3 H), 3.54-3.43 (four peaks, 1 H), 2.92-2.63 (m, 2 H), 2.08 (q, J = 7.1 Hz, 2 H), 0.83 (t, J = 7.1 Hz, 3 H); referred to the other stereoisomer δ 7.35 (tight m, 5 H), 3.67 (s, 3 H), 3.84-3.73 (four peaks, 1 H), 2.97-2.62 (m, 2 H), 1.98 (q, J = 7.1 Hz, 2 H), 0.82 (t, J = 7.1 Hz, 3 H); IR (film) 3095-3005, 2975-2850, 1780, 1740, 1602, 1450, 1195, 703 cm^{-1}. Anal. Calcd for $C_{14}H_{16}O_4$: C, 67.73; H, 6.49. Found: C, 67.97; H, 6.55.

Kinetic Studies. The relative rate constants of Z- and R-substituted styrenes with respect to styrene in the CAN-promoted oxidative addition of 2,4-pentanedione, methyl 3-oxobutanonate, and dimethyl malonate were determined by the competitive method. Kinetic data for the oxidative addition of dimethyl malonate to Z-substituted styrenes were available from a previous work.[10] To a solution of CAN (1-3 mmol) in 4 mL of solvent, a solution of dicarbonyl compound (ca. 0.5 mmol), substituted styrene (0.6-1.0 mmol), styrene (0.6-1.0 mmol), and m-chlorotoluene (ca. 0.75 mmol) as an internal standard in the same solvent (1.0 mL) was added at 20 °C. The mixture was allowed to react 2-5 min (reactions with 2,4-pentanedione and methyl 3-oxobutanoate) or 40 min (reactions with dimethyl malonate). The mixture was poured into water (50 mL) and extracted with hexane (3 × 25 mL). The collected organic phases were washed with water (75 mL), dried with anhydrous sodium sulfate, and analyzed by GLC after suitable dilution. The relative rate constants were determined by the equation $k_S/k_H = \log (S^\circ/S)/\log (H^\circ/H)$ where S°/S and H°/H are the molar ratios of substituted styrene and styrene, respectively, before and after the reaction. In all cases the amount of reacted styrenes corresponds (5%) to the amount of dicarbonyl compound consumed in the reaction. An identical procedure was followed for the reactions with dimethyl malonate where CAN was used in a 6:1 molar ratio with respect to the dicarbonyl compound, as well as for the reactions carried out in the presence of lithium perchlorate. In the reactions with 2,4-pentanedione, competitive experiments were also carried out using a large calculated excess of styrenes and the relative rates determined by the equation $k_S/k_H = (P_S/P_H)(H^\circ/S^\circ)$. Here, P_S/P_H is the molar ratio of the reaction products formed from the competing styrenes and H°/S° is the molar ratio between unsubstituted and substituted styrene before the reaction. In no cases the difference between the values determined by the two methods exceeded 3%.

Acknowledgment. We are very grateful to Prof. B. Giese for making available a manuscript to us before publication. We also thank the Ministero della Universita' e della Ricerca Scientifica e Tecnologica and the Consiglio Nazionale delle ricerche (C.N.R.) for financial support.

Registry No. 5b, 2039-93-2; **5c**, 17498-71-4; **5d**, 5676-29-9; **8** (R = R′ = Me, R″ = H, Ar = C_6H_5), 13463-61-1; **8** (R = R′ = Me, R″ = H, Ar = p-MeC_6H_4), 134391-03-0; **8** (R = R′ = Me, R″ = H, Ar = p-ClC_6H_4), 134391-04-1; **8** (R = R′ = Me, R″ = H, Ar = m-ClC_6H_4), 134391-05-2; **8** (R = R′ = Me, R″ = H, Ar = m-$NO_2C_6H_4$), 134391-06-3; **8** (R = R′ = R″ = Me, Ar = C_6H_5), 54023-32-4; **8** (R = R′ = Me, R″ = Et, Ar = C_6H_5), 134391-07-4; **8** (R = R′ = Me, R″ = i-Pr, Ar = C_6H_5), 134391-08-5; **8** (R = R′ = Me, R″ = t-Bu, Ar = C_6H_5), 134391-09-6; **8** (R = Me, R′ = OMe, R″ = H, Ar = C_6H_5), 134391-10-9; **8** (R = Me, R′ = OMe, R″ = H, Ar = m-$NO_2C_6H_4$), 134391-11-0; **9** (R = R′ = OMe, R″ = i-Pr, Ar = C_6H_5), 134391-12-1; **9** (R = R′ = OMe, R″ = t-Bu, Ar = C_6H_5), 134391-16-5; **9** (R = R′ = OMe, R″ = Me, Ar = C_6H_5), 134391-18-7; **9** (R = R′ = OMe, R″ = Et, Ar = C_6H_5), 134391-21-2; **10** (R′ = OMe, R″ = i-Pr, Ar = C_6H_5) (isomer 1), 134391-13-2; **10** (R′ = OMe, R″ = i-Pr, Ar = C_6H_5) (isomer 2), 134391-14-3; **10** (R′ = OMe, R″ = t-Bu, Ar = C_6H_5), 134391-17-6; **10** (R′ = OMe, R″ = Me, Ar = C_6H_5) (isomer 1), 134391-19-8; **10** (R′ = OMe, R″ = Me, Ar = C_6H_5) (isomer 2), 134391-20-1; **10** (R′ = OMe, R″ = Et, Ar = C_6H_5) (isomer 1), 134391-22-3; **10** (R′ = OMe, R″ = Et, Ar = C_6H_5) (isomer 2), 134391-23-4; CAN, 15078-94-1; α-methylstyrene, 98-83-9; ethyl phenyl ketone, 93-55-0; isopropyl phenyl ketone, 611-70-1; *tert*-butyl phenyl ketone, 938-16-9; methyltriphenylphosphonium iodide, 2065-66-9; lithium diphenylcuprate, 23402-69-9; pivaloyl chloride, 3282-30-2; 2,4-pentanedione, 123-54-6; p-methylstyrene, 622-97-9; styrene, 100-42-5; p-chlorostyrene, 1073-67-2; m-chlorostyrene, 2039-85-2; m-nitrostyrene, 586-39-0; methyl 3-oxobutanoate, 105-45-3; 5-isopropyl-5-phenyl-γ-butyrolactone, 134391-15-4.

Stereochemical Control of Microbial Reduction. 17. A Method for Controlling the Enantioselectivity of Reductions with Bakers' Yeast

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Received September 25, 1990 (Revised Manuscript Received April 13, 1991)

The stereoselectivity of the bakers' yeast catalyzed reduction of β-keto esters to optically active β-hydroxy esters can be controlled by the introduction of a third reagent. To gain insight into the mechanism of this enzymatic reduction, β-hydroxy ester oxidoreductases were isolated from the cells of raw bakers' yeast. Four dominant competing enzymes were isolated, purified, and characterized. Among these, two reduce β-keto esters stereospecifically to the corresponding D-β-hydroxy esters. The other two afford the L-hydroxy esters. The rates of enzymatic reduction were determined in the presence and absence of the additives.

Introduction

The development of methods for the synthesis of optically active compounds has become one of the most important goals in the fields of organic chemistry and biochemistry. In recent years, there have been dramatic developments in the asymmetric synthesis of organic compounds.[1] In particular, biological methods have been extensively developed within the last decade.[2]

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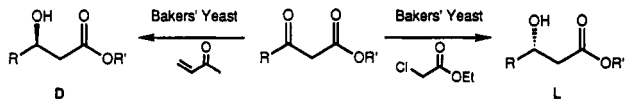
0022-3263/91/1956-4778$02.50/0

Scheme I



Bakers' yeast reduces various β-keto esters to the corresponding optically active β-hydroxy esters. These compounds can be utilized as chiral building blocks in the synthesis of natural products.[3] However, the stereoselectivity of such reductions is not always high, and a particular microbe does not necessarily afford a product of the desired configuration. A number of researchers have tried to control and improve the stereoselectivity of the reduction by screening microbes,[4] modifying the structure of the substrate,[5] and modifying the reaction conditions.[6]

Recently, we reported a quite new and useful method of reduction with bakers' yeast.[7-9] We found that the introduction of a third reagent into the reaction system changes the stereoselectivity of the reduction and allows a product of the desired configuration to be obtained in high enantiomeric excess. Thus, the introduction of allyl alcohol[7] or an α,β-unsaturated carbonyl compound[8] shifts the stereoselectivity of the reduction toward the D isomer, whereas the introduction of ethyl chloroacetate[9] favors the formation of the L isomer (Scheme I). The method is useful because the stereoselectivity can be easily controlled without screening microbes or modifying the structure of the substrate.

Because it would be valuable to elucidate the mechanism by which the stereoselectivity of microbial reduction is controlled by an achiral additive, several β-hydroxy ester oxidoreductases were isolated from the cells of raw bakers' yeast and the effects of additives on the activities of the enzymes were studied. The rates of enzymatic reduction were determined in the presence and absence of the additives. The degree of stereochemical control was substantiated by the results of the rate studies.

Experimental Section

Chromatography. Analytical GLC was performed with a 25-m PEG-20M Bonded column at 130 °C. Preparative GLC was performed at 150 °C with a 1.5-m PEG-20M column. HPLC was performed with a 4.6 mm × 150 mm Wakosil 5SIL column.

Materials. Ethyl 4-chloro-3-oxobutanoate was purchased from Tokyo Kasei Kogyo Co. (*R*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) was purchased from Aldrich Chemical Co. Bakers' yeast and a MW-Marker (a mixture of cytochrome c, 12.4 kDa; adenylate kinase, 32 kDa; enolase, 67 kDa; lactate dehydrogenase, 142 kDa; glutamate dehydrogenase, 290 kDa) were purchased from Oriental Yeast Co. DEAE-Toyopearl 650 and Butyl-Toyopearl 650 were purchased from Tosoh Co. The Superose 12 column was obtained from Pharmacia Fine Chemicals. The Ultra Pack PEI (polyethylene imine), CE (carboxy ethyl), and HIC (HI-propyl) columns were purchased from Yamazen Co. Cellurofine GLC 2000 was purchased from Seikagaku Kogyo Co. Asahipack GS-510 was obtained from Asahi Chemical Industries Co. CentriCell ultrafilters (10000 NMWL) were purchased from Polysciences, Inc. Other reagents were purchased from Nacalai Tesque Co.

The basic buffer consisted of 10 mM potassium phosphate (pH 7.00), 0.05% 2-mercaptoethanol, 1 mM dithiothreitol, and 1 mM (phenylmethyl)sulfonyl fluoride.

Enzyme Assay. A 50-μL aliquot of chromatographic fraction (vide infra) was added to 3.00 mL of a solution of 0.10 M potassium phosphate buffer (pH 7.0) that also contained ethyl 4-chloro-3-oxobutanoate (0.98 mM) and NADPH (0.09 mM). The rate of reaction was determined spectrophotometrically at 30 °C by following the decrease in absorbance of NADPH at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADPH per minute at 30 °C under the these conditions.

Determination of Optical Purity of the Product. To a stirred solution of the product β-hydroxy ester (0.03 mmol) and benzene (1 mL) were added, in order, pyridine (1.5 mmol) and (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (0.2 mmol). The mixture was stirred overnight. EtOAc and H_2O was then added. The two liquid layers were separated. The organic layer was washed (1 M aqueous HCl, saturated aqueous Na_2CO_3, and brine), dried (Na_2SO_4), and concentrated to give the corresponding (*R*)-MTPA ester in more than 95% yield in all instances.

The enantiomeric excess (ee) of the hydroxy ester was determined by HPLC (hexane–EtOAc, 30:1) analysis of the corresponding (*R*)-MTPA ester.

Purification of the β-Hydroxy Ester Oxidoreductases. The enzymes were isolated, purified, and stored at 4 °C. For prolonged storage, the enzymes were frozen in 10% aqueous glycerol. Thus, raw bakers' yeast (1 kg) was suspended in 10 mM phosphate buffer (2 L). The suspended cells were disrupted with a Dyno-Mill operating at a flow rate of 30 mL/min. The disrupted mixture was centrifuged at 100000*g* for 30 min. The supernatant liquid (1.4 L) was dialyzed overnight against 10 mM phosphate buffer (10 L). The buffer was renewed, and dialysis was continued overnight. The dialyzate was concentrated by dialysis against poly(ethylene glycol) (MW 20000) overnight. The concentrated dialyzate (500 mL) was then centrifuged at 100000*g* for 30 min to obtain 400 mL of supernatant liquid.

To eliminate nucleic acids and noneffuse proteins, the cell-free solution (400 mL) was applied to a 6 cm × 30 cm column packed with DEAE-Toyopearl 650 equilibrated with the basic buffer. The proteins were eluted with the basic buffer, which also contained 0–0.4 M linear gradient of KCl (4 L). The active fractions were collected and concentrated to 250 mL by dialysis against poly(ethylene glycol) (MW 20000). The concentrated enzyme solution was then dialyzed for 1 day against 3 L of the basic buffer with frequent renewal of the buffer.

The dialyzate was applied to a 6 cm × 30 cm column packed with DEAE-Toyopearl 650 equilibrated with the basic buffer. The column was eluted with the basic buffer (2 L) at a flow rate of 2.0 mL/min. A nonadsorbed enzyme (L-enzyme-1) was eluted. Then, elution was continued with a 0–0.3 M linear gradient concentration of KCl dissolved in the basic buffer (5 L). Fractions of 20 mL were collected. Three enzymes were eluted: at 0.04 M (D-enzyme-1), 0.06 M (L-enzyme-2), and 0.15 M (D-enzyme-2) KCl. These active fractions were each concentrated to 80 mL by dialysis against poly(ethylene glycol) (MW 20000).

Each of the four enzyme solutions was then applied to a 3 cm × 40 cm column packed with Butyl-Toyopearl 650 equilibrated with the basic buffer, which also contained 20% $(NH_4)_2SO_4$. The column was eluted with the basic buffer, which also contained

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20% $(NH_4)_2SO_4$, and then with a 20–0% linear gradient concentration of $(NH_4)_2SO_4$ dissolved in the basic buffer (400 mL). Fractions of 9.0 mL were collected. Those that exerted activity were combined and concentrated to 0.5 mL by ultrafiltration with a CentriCell centrifugal ultrafilter (10000 NMWL). Then, the D-enzymes were directly subjected to gel filtration column chromatography to determine their molecular weights and, at the same time, to purify them for rate studies.

The solution of L-enzyme-1 or L-enzyme-2 was applied to an Ultra Pack PEI column equilibrated with the basic buffer. The column was eluted with a 0–0.5 M linear gradient concentration of NaCl dissolved in the basic buffer. Fractions that exerted activity were combined and concentrated to 3 mL by ultrafiltration.

The concentration solution of L-enzyme-1 was applied to an Ultra Pack CE column equilibrated with the basic buffer (pH 6.0). The column was eluted with a 0–0.5 M linear gradient concentration of NaCl dissolved in the same buffer. Fractions that exerted activity were combined and concentrated to 1 mL by ultrafiltration.

The concentration solution of L-enzyme-2 was applied to an Ultra Pack HIC column equilibrated with the basic buffer, which also contained 20% $(NH_4)_2SO_4$. The column was eluted with a 20–0% linear gradient concentration of $(NH_4)_2SO_4$ dissolved in the basic buffer. Fractions that exerted activity were combined and concentrated to 1 mL by ultrafiltration.

The solution of L-enzyme-1 or L-enzyme-2 so obtained was applied to a gel filtration column (Cellurofine GLC 2000 equilibrated with the basic buffer which also contained 0.1 M KCl). The column was eluted with the same buffer. Fractions that exerted activity were combined and concentrated to 0.1 mL by ultrafiltration.

Determination of the Molecular Weights of the Enzymes. A solution of D-enzyme-1, D-enzyme-2, or the MW-Marker was applied to a gel filtration column (Superose 12 equilibrated with the basic buffer, which also contained 0.1 M KCl). After elution with the same buffer (flow rate 0.5 mL/min), the activity of each fraction was assayed as described previously. The molecular weights of D-enzyme-1 and D-enzyme-2 were found to be 25 and 1600 kDa, respectively.

A solution of L-enzyme-1, L-enzyme-2, or the MW-Marker was applied to a gel filtration column (Asahipak GS-510 equilibrated with the basic buffer, which also contained 0.1 M KCl). After elution with the same buffer (flow rate 0.5 mL/min), the activity of each fraction was aasayed as described previously. The molecular weight of both L-enzyme-1 and L-enzyme-2 was found to be 32 kDa.

Enzymatic Reduction of Ethyl 4-Chloro-3-oxobutanoate. In a glass reaction vessel were placed a concentrated solution of the enzyme (10 units), NADPH (10 mg), G6PDH (glucose 6-phosphate dehydrogenase, 50 units, 0.3 mg), G6P (glucose 6-phosphate, 300 mg), ethyl 4-chloro-3-oxobutanoate (82 mg, 0.5 mmol), and 0.1 M phosphate buffer (pH 7.0, 30 mL). The reaction vessel was shielded from light. The mixture was magnetically stirred for 24 h at 30 °C. Hyflo Super-Cel and EtOAc were added. The mixture was filtered. The solid that was collected was washed with EtOAc. The combined washings and filtrate were washed (H_2O and brine), dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by preparative gas chromatography (1.5-m PEG column, 160 °C) to give ethyl 4-chloro-3-hydroxybutanoate. The yields of products were 62, 80, 45, and 84% from the reductions catalyzed by L-enzyme-1, L-enzyme-2, D-enzyme-1, and D-enzyme-2, respectively. The optical purity and stereochemistry of the products were determined by the method mentioned previously. The ee of the products were, in all instances, more than 99%.

Results

Purification and Properties of β-Hydroxy Ester Oxidoreductases. The β-hydroxy ester oxidoreductases were isolated from a cell-free solution of bakers' yeast by anion exchange column chromatography (DEAE-Toyopearl). A typical chromatogram is shown in Figure 1. Each enzyme was further purified by chromatographic methods, which included hydrophobic chromatography and gel filtration chromatography.

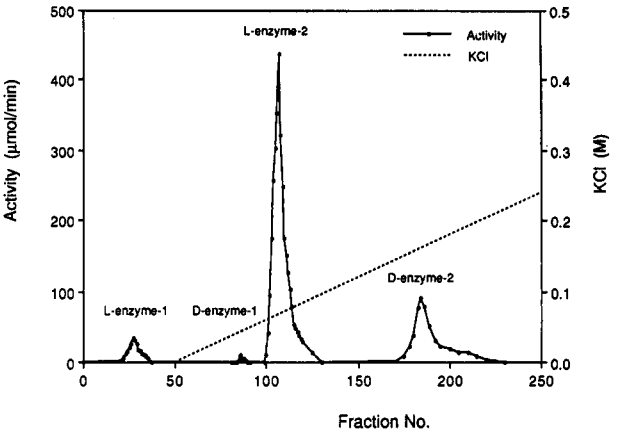

Figure 1. Elution chromatogram of β-hydroxy ester oxidoreductases derived from bakers' yeast obtained with DEAE-Toyopearl.

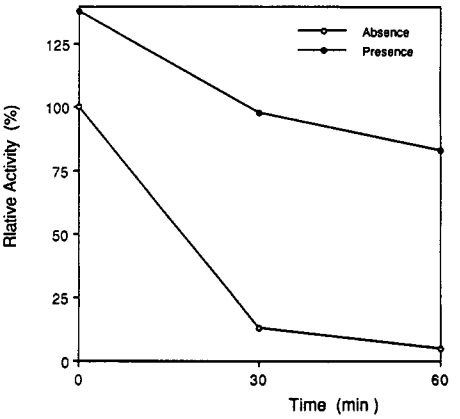

Figure 2. Stability of L-enzyme-2 in the absence of and presence of bovine serum albumin (BSA).

Table I. Enzymatic Reduction of Ethyl 4-Chloro-3-oxobutanoate

enzyme	M_W (kDa)	total activity (U)	config of the product	ee (%)
D-enzyme-1	25	26	D	>99
D-enzyme-2	1600	1442	D	>99
L-enzyme-1	32	246	L	>99
L-enzyme-2	32	3108	L	>99

Each of the enzymes preferentially utilizes NADPH as the coenzyme. The total activities of the enzymes are summarized in Table I.

The results demonstrate that D-enzyme-1 contributes little (ca. 0.5%) to the reduction. It would be reasonable, therefore, to assume that almost all of the reduction by bakers' yeast is catalyzed by D-enzyme-2, L-enzyme-1, and L-enzyme-2. Hence, only the rates of reduction catalyzed by the three latter were determined. The stereospecificity of each enzymatic reduction was established by reducing ethyl 4-chloro-3-oxobutanoate, in the presence of a G6P (glucose 6-phosphate)–G6PDH (glucose 6-phosphate dehydrogenase) system in order to regenerate NADPH from $NADP^+$.[10] The enantiomeric excess (ee) of the product was determined by HPLC analysis of the corresponding (*R*)-MTPA ester. L-Enzyme-2 is too labile to yield the product hydroxy ester under the reaction conditions employed (30 °C), whereas, under the same conditions, the

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Table II. Kinetic Constants of Reductions Catalyzed by the Enzymes[a]

enzyme	K_M (mM)	$k_{cat.}$ (s^{-1})
D-enzyme-1	0.59	
D-enzyme-2	4.54	303[b]
L-enzyme-1	0.13	6.20
L-enzyme-2	0.15	4.66

[a] Errors in K_M and $k_{cat.}$ are estimated to be less than ±10%. [b] Reference 10.

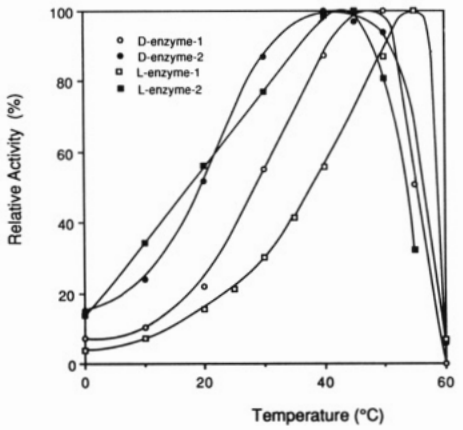

Figure 3. Effect of temperature on the activity of the enzymes.

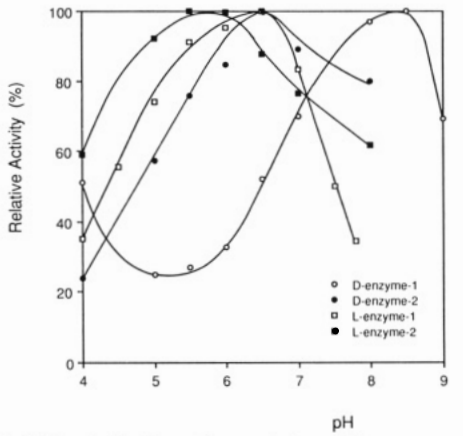

Figure 4. Effect of pH on the activity of the enzymes.

other enzymes do catalyze the reduction and produce the hydroxy ester. However, as shown in Figure 2, L-enzyme-2 can be stabilized by bovine serum albumin (BSA). Thus, in the presence of BSA, L-enzyme-2 is able to afford the product, and, furthermore, the rate of reduction could be determined.

The data listed in Table I show that all four enzymes reduce β-keto esters quantitatively and stereoselectively.

The molecular weights of the enzymes, as determined by gel filtration column chromatography, are also listed in Table I. It should be noted that the value of the experimentally determined molecular weight of D-enzyme-2 is accompanied by an experimental error because the exclusion limit of the gel filtration column is 300 kDa.

To further characterize the enzymes, the rates of enzymatic reduction were determined. The results are listed in Table II. The effects of temperature and pH on the enzymatic activity and the thermal stability of each of the enzymes were also determined. The results are shown in Figures 3–5, respectively. The results yielded fundamental information on how to control the stereoselectivity of the reduction by modifying the reaction conditions.

The L-enzymes possess similar properties, e.g., molecular weight and stereospecificity, K_M and k_{cat}. However, they

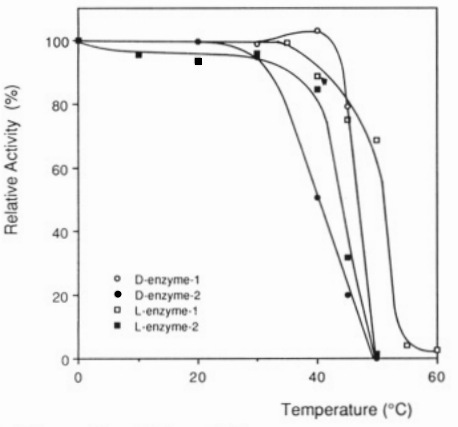

Figure 5. Thermal stability of the enzymes.

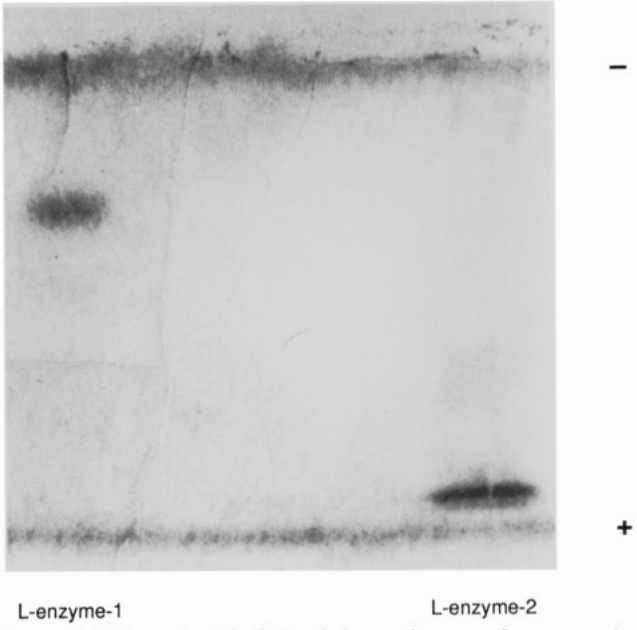

Figure 6. Polyacrylamide disk gel electrophoreses of L-enzyme-1 and L-enzyme-2.

behaved differently during anion-exchange column chromatography. To differentiate the two enzymes, the homogeneity of each was determined by polyacrylamide disk gel electrophoresis.[11] As Figure 6 shows, it is apparent that the two possess quite different electrostatic properties: the surface of L-enzyme-1 is more positively charged than that of L-enzyme-2.

Effect of Additives. Bakers' yeast catalyzes the reduction of ethyl 4-chloro-3-oxobutanoate to the corresponding β-hydroxy ester. The stereoselectivity of the reduction, however, is low[5e,8] because the D- and L-enzymes of bakers' yeast display nearly equal activities toward the substrate. It is conceivable that the change in stereoselectivity that occurs on introduction of an additive stems from an interaction between the additive and one or more of the oxidoreductases. To gain information on how additives affect the stereoselectivity of the reduction, the activity of each enzyme was measured in the presence of varying amounts of such additives as methyl vinyl ketone and ethyl chloroacetate. The reaction is inhibited by such compounds. The inhibition patterns were determined from the Dixon plots (plots of $1/v$ vs [I]) shown in Figures 7 and 8. The results show that the mechanism of inhibition by methyl vinyl ketone is noncompetitive for all the

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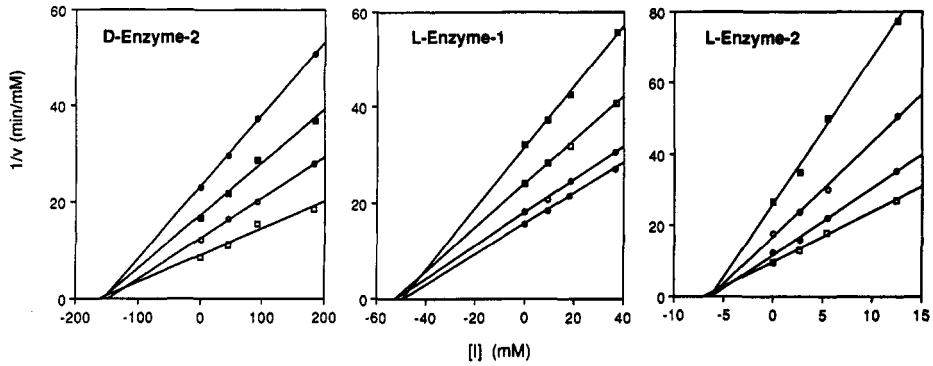

Figure 7. Dixon plots for the inhibition by methyl vinyl ketone.

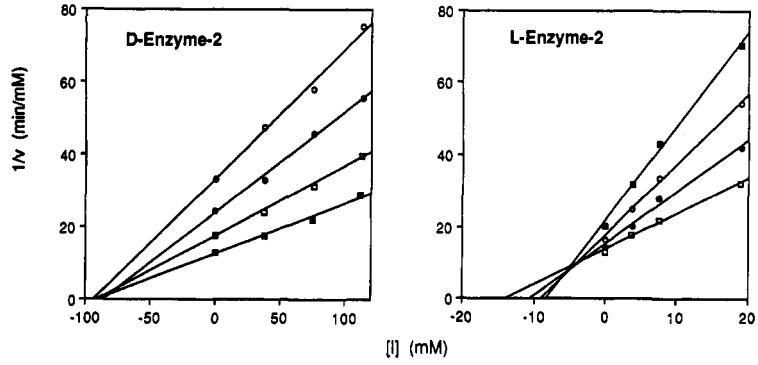

Figure 8. Dixon plots for the inhibition by ethyl chloroacetate.

Table III. Inhibition Constants and Inhibition Patterns of the Enzymes

inhibitor	enzyme	K_i[a] (mM)	inhibition pattern
methyl vinyl ketone	D-enzyme-2	154	noncompetitive
	L-enzyme-1	50.6	noncompetitive
	L-enzyme-2	6.38	irreversible[b]
ethyl chloroacetate	D-enzyme-2	89.2	noncompetitive
	L-enzyme-1	very large	
	L-enzyme-2	4.42	mixed

[a] Errors in K_i are estimated to be less than ±10%. [b] Irreversibility was confirmed only for this enzyme. Partial irreversibility was observed with the other enzymes. However, no definite conclusions could be drawn.

enzymes and that by ethyl chloroacetate is also noncompetitive for D-enzyme-2 but is of a mixed type for L-enzyme-2. The activity of L-enzyme-1 is little affected, even in solutions saturated with ethyl chloroacetate (ca. 150 mM). The kinetic constants obtained from the Dixon plots and the inhibition patterns are shown in Table III.

Discussion

There are several oxidoreductases present in bakers' yeast. For example, Furuichi et al.[12] isolated a β-hydroxy ester oxidoreductase therefrom. The purified enzyme catalyzes the reduction of benzyl 2-methyl-3-oxobutanoate to the corresponding L-β-hydroxy ester, and thus is an L-enzyme. There is no doubt, however, that this enzyme differs from the L-enzymes described here, because the latter catalyze the reduction of ethyl 2-methyl-3-oxobutanoate,[13] whereas the former does not. Another oxidoreductase was isolated by Furuichi et al.[14] from *Saccharomyces fermentati*. This enzyme also differs from the L-enzymes described here, both in molecular weight and substrate specificity.[15] Shieh et al.[16] isolated three dominant competing β-hydroxy ester oxidoreductases from bakers' yeast. All three catalyze the reduction of ethyl 4-chloro-3-oxobutanoate. Studies[16] of the stereospecificity of the reductions catalyzed by these enzymes revealed that two, one of which was attributable to fatty acid synthetase (FAS), give the D-hydroxy ester, and thus are D-enzymes, whereas the thrid gives the L-hydroxy ester, and thus is an L-enzyme. The D-enzyme-2 described here may be identical with the FAS of Shieh et al. because the stereospecificities, molecular weights, and kinetic constants of the two are very similar. The other D-enzyme described by Shieh et al. also has a molecular weight and kinetic constants similar to the D-enzyme-1 described here. Thus, the latter two enzymes may be the one and the same. However, the L-enzyme of Shieh et al. is distinct from the L-enzymes described here. Why this is so is not clear. It may reflect the fact that the samples of bakers' yeast used in the two studies were obtained from different sources.

Because the presence of α,β-unsaturated carbonyl compounds leads to an increase in the yield of the D product and also retards the rate of reduction,[8] there is no doubt that such compounds inhibit the activities of the L-enzymes. The results of rate studies, shown in Figure 7 and listed in Table III, clearly support this conclusion. An expression for the relative rate of reduction (a) in the presence of a noncompetitive inhibitor is[17]

$$a = K_i/(K_i + [I])$$

Under preparative conditions, where the concentration of methyl vinyl ketone is ca. 50 mM, the relative rates of reduction catalyzed by D-enzyme-2, L-enzyme-1, and L-enzyme-2 are 0.75, 0.50, and 0.11, respectively. In other words, when the sum of the activities of the D- and L-enzymes is taken into consideration, it can be concluded that, in the presence of methyl vinyl ketone, the D-enzyme retains 75% of its original activity, whereas the L-enzymes

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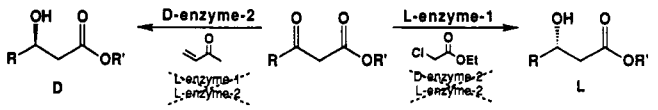
Scheme II



retain only 14%. Thus, D-enzyme-2 is virtually solely responsible for the catalysis of the reduction. Consequently, the D product is afforded stereoselectively. The introduction of an alkyl α-haloacetate shifts the stereoselectivity of the reduction toward formation of the L product.[9] It is believed, therefore, that such compounds inhibit the activity of D-enzyme. However, as the results shown in Figure 8 and listed in Table III demonstrate, ethyl chloroacetate inhibits not only the activity of D-enzyme-2 but also that of L-enzyme-2. On the other hand, the activity of L-enzyme-1 is unaffected by this additive. So, it can be concluded that, in the presence of ethyl chloroacetate, the reduction by bakers' yeast is catalyzed only by L-enzyme-1.

Of course, the results obtained from in vitro studies do not satisfactorily account quantitatively for the results obtained in vivo. Because the location of the enzymes within the yeast cell, the permeability of the cell wall toward organic reagents, and many other complex biological phenomena must be taken into consideration to understand the behavior of living microbes, the difference in the results of in vitro and in vivo studies is not surprising. Nevertheless, at least qualitatively, it is apparent that the activities of the enzymes of bakers' yeast are specifically inhibited by certain compounds. Thus, the stereospecificity of reductions by bakers' yeast can be changed. The effects of additives on the stereoselectivity of the reduction can be summarized by the reactions shown in Scheme II.

Studies of the enzymatic reduction of other substrates and the application of the purified enzymes described here to organic synthesis are now in progress. The results will be reported elsewhere.

Acknowledgment. We thank the Ministry of Education, Science and Culture for financial support (Grants-in-Aid No. 01470022, 01303007, and 02303004).

Registry No. D-β-keto ester reductase, 114705-02-1; L-α-keto ester reductase, 114705-03-2; ethyl 4-chloro-3-oxobutanoate, 78-94-4; ethyl chloroacetate, 105-39-5.

Calixarenes. 25. Conformations and Structures of the Products of Arylmethylation of Calix[4]arenes

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Received June 25, 1990

The arylmethylations of calix[4]arenes reported in this paper serve as companion pieces to an earlier study of the effects of reaction conditions and the structure of the derivatizing agent on the conformational outcome of the aroylation of calix[4]arenes. In contrast to the aroylation reaction, where a significant and predictable relation is observed between the para substituent of the aroyl chloride and the ratio of conformers formed, the arylmethylation reaction shows only a small and much less easily predictable dependence of the conformer ratio on the para substituent of the arylmethyl halide. Also, whereas the products of aroylation are the cone and/or 1,3-alternate conformers, those of arylmethylation are the cone and/or partial cone conformers. While no rationale has yet emerged to explain this difference, a study of the benzylation of dibenzyl and tribenzyl ethers of 5,11,17,23-tetra-*tert*-butyl-25,26,27,28-tetrahydroxycalix[4]arene has established that the conformations of the tetraethers are not completely established until the third step in some cases and the fourth step in others.

Calixarenes are conformationally mobile macrocyclic compounds that are the focus of considerable attention because of their ability, when appropriately functionalized, to serve as ionophores and enzyme mimics.[1] The possibility for conformational isomerism in the calix[4]arenes was first recognized by Megson[2] and Ott and Zinke;[3] the concept was sharpened by Cornforth and co-workers;[4] and the process of conformational interconversion has now been studied in detail by several groups of researchers including Kämmerer et al.,[5,6] Munch,[7] Gutsche et al.,[8,9] Shinkai et al.,[10-13] Ungaro et al.,[14] and Reinhoudt et al.[15] The four "up-down" conformers that are possible have been designated as cone, partial cone, 1,2-alternate, and 1,3-alternate, as depicted in Figure 1.

Upon replacement of the phenolic hydrogens with sufficiently large groups, the calix[4]arenes become conformationally inflexible, existing as discrete entities in one or another of the conformations.[16,17] A study of the aroylation of calix[4]arenes carried out in this laboratory[18]

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